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The Tn10 Synaptic Complex Can Capture a Target DNA only after Transposon Excision

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Summary

Tn10 transposes nonreplicatively. Staged in vitro reactions demonstrate that a Tn10 synaptic complex can become committed to a particular target DNA molecule via a noncovalent interaction in the absence of strand transfer. Commitment occurs only after double-strand cleavage at both transposon ends (in "double-end break" [DEB] complexes). Stable noncovalent DEB–target DNA cocomplexes can be detected, but no cocomplexes occur with synaptic complexes containing uncleaved ends. Preincubation of DEB complexes with target DNA accelerates the rate of strand transfer. Postcleavage target capture is remarkable for Tn10; Mu and Tn7 select a target site prior to cleavage. Promiscuous target selection may favor evolution of IS-based composite elements while being suicidal for other types of transposons.

Introduction

Tn10 is a composite transposon comprising two IS10 modules in inverted repeat orientation flanking tetracycline resistance genes (Kleckner, 1989; Kleckner et al., 1996). Tn10 transposes via a nonreplicative mechanism: the element is excised from the donor molecule by flush double-strand breaks at its two ends, which are then joined to target DNA (Bender and Kleckner, 1986; Haniford et al., 1989; Benjamin and Kleckner, 1992; Chalmers and Kleckner, 1996).

At each transposon end, the three chemical steps of the transposition reaction occur in a defined order. First, a nick occurs on the transferred strand (the strand that is eventually transferred to the target DNA). Then, the nontransferred strand is nicked, giving double-strand cleavage. Finally, strand transfer occurs, subsequent to and dependent upon double-strand cleavage (Haniford et al., 1991; Bolland and Kleckner, 1995; Chalmers and Kleckner, 1996); the two 3′ OH termini likely attack the target DNA directly in a single-step transesterification reaction, as is the case for Mu bacteriophage and retrovirus HIV (Engelman et al., 1991; Mizuuchi and Adzuma, 1991; Kleckner et al., 1996).

The chemical steps of Tn10 transposition all occur within a stable synaptic complex containing the two transposon ends (Haniford et al., 1991; Sakai et al., 1995), as is the case for other well-studied elements (Mizuuchi, 1992; Grindley and Leschziner, 1995; Lavoie and Chaconas, 1996). These synaptic complexes, or transpososomes (Surette et al., 1987), coordinate events at the two transposon ends. Tn10 transposition begins with the assembly of a "paired-ends complex" (PEC), in which the two ends are uncleaved. In later forms, the transposon ends have undergone double-strand cleavage at one or both ends ("single-end break" [SEB] complexes and "double-end break" [DEB] complexes) or double-end cleavage plus strand transfer to a target DNA (Haniford et al., 1991; Sakai et al., 1995; this work). As for Mu transpososomes, changes in the DNA components are accompanied by important transitions in the protein–DNA complex as a whole (Haniford et al., 1991; Sakai et al., 1995).

At some point, target DNA must enter the transposition reaction. Only two transposons have been studied previously in this regard, Mu and Tn7. In the transposition reactions of both elements, target DNA enters the reaction very early, prior to end cleavage. Moreover, target entry is mediated in each case by a transposon-encoded target DNA-specific binding protein, MuB and TnsD, respectively. Tn7 transposition by the TnsD pathway absolutely requires appropriate target DNA for the coassembly of protein components and transposon ends into a stable complex prior to the first chemical step (Bainton et al., 1993). For Mu, the chemical steps are not absolutely dependent upon prior interaction with target DNA (Craigie and Mizuuchi, 1987), but a complex between MuB and target DNA acts as a potent allosteric effector of strand nicking within the precleavage synaptic complex (Baker et al., 1991; Mizuuchi et al., 1992); preincubation of uncleaved synaptic complexes with MuB–target DNA complexes dramatically reduces the time required for cleavage, from ~60 min to ~2 min. Thus, in actual fact, effective interaction of uncleaved synaptic complexes with target DNA will almost always precede and provoke strand cleavage.

The experiments presented here investigate entry of target DNA into the Tn10 transposition reaction. We examined functional and physical interactions between target DNA and Tn10 synaptic complexes representing different stages of the transposition reaction prior to strand transfer (i.e., PECs, SEBs, and DEBs). Using staged in vitro reactions, we asked whether and which synaptic complex(es) can become committed to a particular target DNA prior to strand transfer, we examined the formation and dissociation of synaptic complex–target DNA cocomplexes by a gel comigration assay, and we asked whether formation of a noncovalent cocomplex between a transpososome and a target DNA is rate determining for formation of a covalent strand-transfer product.

The results presented suggest that target DNA only enters the Tn10 transposition reaction at a late stage, after double-strand cleavage at the transposon ends. Reasons why Tn10 differs from Mu and Tn7 in this regard and mechanistic aspects of transpososome–target DNA interactions are discussed.

Results

Stable Commitment of a Synaptic Complex to a Target DNA

Assay for Target Commitment

The ability of a particular type of Tn10 synaptic complex to become "committed" to a specific individual target

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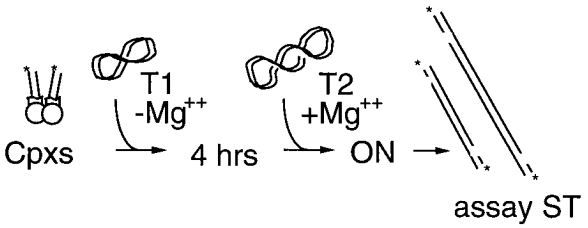

Figure 1. Two-Stage Target Commitment Assay

Synaptic complexes incorporating transposase and radiolabeled (asterisk) transposon-end DNA fragments are assembled in the absence of Mg^{2+} (DEB complexes depicted). Stage I: complexes are incubated with a particular supercoiled plasmid target DNA (T1) for 4 hr to permit interactions between the 2 species to come to equilibrium. Unless otherwise specified, 4 mM $CaCl_2$ is present. Stage II: a second target DNA of distinguishable size (T2) is added. Simultaneously, the reaction is adjusted to 4 mM $MgCl_2$, which permits strand transfer to begin. The mixture is incubated overnight to permit completion of strand transfer. Reactions are stopped, proteins removed by phenol extraction, and strand-transfer products to the 2 target molecules are detected in a 1% agarose gel as radiolabeled linear fragments of the two appropriate sizes. An excess of strand-transfer products to T1 as compared to T2 indicates that a functionally significant interaction between synaptic complexes and target DNA occurred during Stage I (see text). The relative numbers of the two types of target DNAs are given by a control reaction in which both are added during Stage I and only Mg^{2+} is added in Stage II.

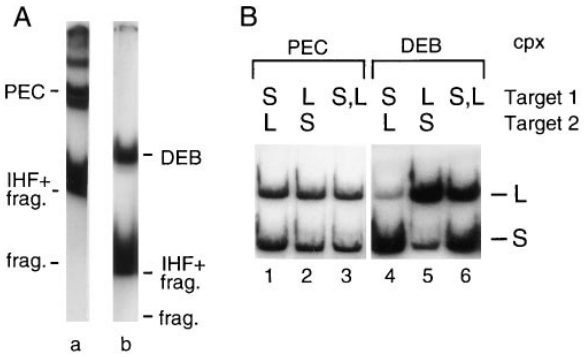

Figure 2. Target Commitment

(A) Representative PEC and DEB assembly mixtures. In lane (b), the DEB complexes are the only transposase-containing species. In lane (a), most PEC complexes occur in the band indicated as "PEC." The small minority of complexes that migrate just above the PEC band comprise an alternative conformer that is just as active as the majority PEC species (Sakai, 1996; see also Figure 4).

(B) PEC (lane 1–3) and DEB (lane 4–6) complexes assayed for target commitment (see Figure 1) directly in assembly mixtures; data are for Experiment V of Table 1. Target DNAs are either a smaller 3 kb plasmid (S) or a larger 5 kb plasmid (L).

DNA molecule is assayed by a two-stage protocol (Figure 1). Stage I: synaptic complexes are incubated for several hours with a large molar excess of supercoiled plasmid target DNA (Target 1) under conditions where strand transfer cannot occur (in the absence of Mg^{2+}). Ca^{2+} is usually present at this stage, for reasons discussed below. During this incubation, interactions between the synaptic complexes and Target 1 should equilibrate. Stage II: a second target DNA of distinguishable size (Target 2) is added to the reaction in an amount equal to Target 1. Target 2 provides a challenge to any synaptic complex–target DNA interactions established during Stage I. Simultaneously, Mg^{2+} is added to initiate strand transfer. Reactions are then incubated further to permit full strand transfer. Under these conditions, nearly 100% of synaptic complexes undergo strand transfer (Sakai et al., 1995; data not shown). The relative frequency of strand transfer to the two target DNAs is then determined. If Target 1 is used in preference to Target 2, it can be inferred that "target commitment" has occurred; that is, a functionally significant interaction between synaptic complexes and target DNA has occurred during Stage I and then persisted into Stage II. A reaction in which both target DNAs are added during Stage I, and only $MgCl_2$ is added in Stage II, allows direct evaluation of the relative numbers of the two types of target DNA molecules in the experiment and potential side effects of Mg^{2+} or higher target DNA concentration.

This experiment permits quantitative evaluation of the percentage of synaptic complexes that, during Stage II, undergo double-end strand transfer to the Target 1 molecule with which they were associated at the end of Stage I ("percent target commitment" [%TC]) (see Experimental Procedures). This quantitation assumes that synaptic complexes that select a target DNA for strand transfer during Stage II choose randomly between the two available types of target molecules.

PEC, SEB, and DEB complexes were all assayed for target commitment. All three types of complexes were assembled in vitro on short linear substrate DNA fragments carrying transposon-end sequences in reactions containing transposase and IHF (Sakai et al., 1995; see Experimental Procedures). PEC complexes were assembled using a standard 136 bp substrate containing 87 bp of the IS10 outside end and 49 bp of flanking donor DNA. DEB complexes were assembled directly on substrate fragments precleaved by a restriction enzyme at bp1 of the transposon but were otherwise identical to those used for PEC complexes. SEB complexes contain one fragment of each type and were prepared from a mixture of the two. DEB and SEB complexes were assembled using a precleaved end(s) in order to avoid use of Mg^{2+} prior to Stage II of the target commitment protocol. Conversion of PEC complexes to SEB and thence to DEB complexes via the normal transposition reaction is absolutely dependent upon Mg^{2+}, but complexes will assemble efficiently on uncleaved or precleaved ends in the absence of divalent metal ion (Sakai et al., 1995; Sakai, 1996). DEB and SEB complexes formed on precleaved ends are just as active as those generated via a transposition reaction (Sakai et al., 1995; Sakai, 1996).

The two target DNAs are supercoiled plasmids of 3 and 5 kb, respectively, and are isogenic but for a 2 kb insert (see Experimental Procedures). Strand transfer of both component transposon ends within a synaptic complex to a circular target molecule ("double-end strand transfer" [DEST]) yields, after deproteinization, a 3 or 5 kb linear target DNA having a radiolabeled substrate fragment covalently joined to each end (Figure 1).

Only DEB Complexes Exhibit Target Commitment

To avoid unnecessary physical manipulation, DEB and PEC complexes were assayed directly in their respective assembly reaction mixtures. In such mixtures, the complexes of interest comprise the major (often the only) transposase–DNA species (Sakai et al., 1995; Sakai, 1996) (Figure 2A); no new complexes form after addition

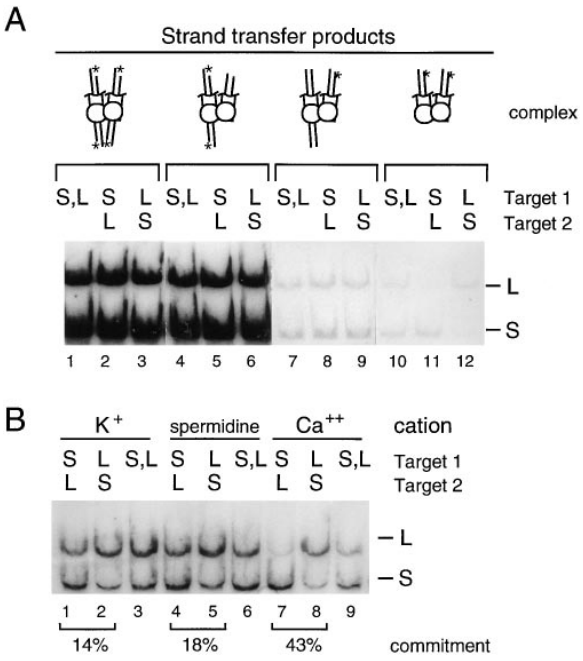

Figure 3. Requirements for Efficient Target Commitment

(A) Purified preparations of synaptic complexes were assayed for target commitment. PEC, lanes 1–3; SEB, label on the standard substrate, lanes 4–6; SEB, label on precleaved substrate, lanes 7–9; DEB, lanes 10–12 (see also [B], Ca^{2+}).

(B) Target commitment assays were carried out with DEB complexes in their assembly mixtures as in Figure 2, except that during Stage I, reactions were either not supplemented (lanes 1–3) or supplemented to 2 mM spermidine (lanes 4–6) or 4 mM $CaCl_2$ (lanes 7–9). Corresponding target commitment levels (see text; Experimental Procedures) were 14%, 18%, and 43%, respectively (average of the results for the 2 orders of addition). Accordingly, the target commitment observed in the K^+ and spermidine conditions was 33% and 42% of that observed in the Ca^{2+} condition.

of target DNA (Sakai et al., 1995). Furthermore, the complexes in these mixtures are extremely active; virtually 100% undergo double-end strand transfer (Sakai et al., 1995).

DEB complexes exhibit robust target commitment. A clear qualitative bias for strand transfer to Target 1 is observed irrespective of which plasmid is added at which stage; the number of Target 1 strand-transfer products exceeds the number of strand-transfer products to Target 2 by a ratio of 3:1 (Figure 2B, lanes 4–6; see also Figure 3 and below; Table 1). Correspondingly, on average 50% of total DEB complexes are inferred to be committed at the end of Stage I (Table 1).

Some type(s) of physical association(s) between a DEB complex and a target DNA molecule must necessarily precede strand transfer. The percentage of DEBs involved in such associations at the end of Stage I must be at least 50% to account for the level of target commitment but could be significantly higher, since the level of commitment is determined not only by the level of DEBs involved in associations but also by the probability that such target-associated DEBs will proceed on to strand transfer during Stage II, rather than dissociating and undergoing another round of target interaction.

PEC complexes, assayed in parallel with DEB complexes, exhibit no detectable commitment. Strand-transfer products to Target 1 and Target 2 occur at equal frequency, with a negligible fraction of complexes committed (Figure 2B, lanes 1–3; see also Figure 3; Table 1).

Table 1. Target Commitment

	Distribution of Strand-Transfer Products[a]		
	Target 1	Target 2	(%TC)[b]
PEC complexes			
Expt I	.48	.52	−0.55
Expt II	.50	.50	0.36
Expt IV	.52	.48	3.5
Expt V	.50	.50	−2.0
Avg.	.50 ± .001	.50 ± .001	0.32
DEB complexes			
Expt II	.70	.30	39
Expt III	.71	.29	42
Expt IV	.78	.22	56
Expt V	.82	.18	62
Avg.	.75 ± .002	.25 ± .002	50

[a] Normalized for input ratio of Target 1 and Target 2; each value is the average of the two values obtained from the two different orders of target addition (i.e., 3 kb > 5 kb and 5 kb > 3 kb). Each standard deviation includes all eight individual values (two values from each of four experiments).

[b] %TC, percentage total complexes committed at Stage I: average for the two different orders of addition (see Experimental Procedures).

SEB complexes must be purified from their more complicated assembly mixture prior to analysis (see Experimental Procedures). Thus, SEB, DEB, and PEC complexes were assembled and gel purified in parallel, and then assayed in parallel for target commitment. As before, DEB complexes exhibit strong target commitment (30%), while PEC complexes exhibit no target commitment (<1%) (Figure 3A, lanes 10–12 and 1–3). SEB complexes also exhibit little or no target commitment (1%) (Figure 3A, lanes 4–9). The lower level of commitment by purified DEB complexes versus unpurified DEBs likely reflects deleterious effects of purification (Sakai, 1996).

Target Commitment Is Enhanced by Divalent Metal Ion

Divalent metal ion is required for the chemical steps of transposition and for normal synaptic complex formation and stability, presumably because the chemical active site also has structural roles (Mizuuchi, 1992; Craig, 1996b). Divalent cation also appears to be very important for Tn10 target commitment. DEB complexes were assayed with varying buffer components at Stage I, i.e., KCl, spermidine, or Ca^{2+}. Target commitment levels were 14%, 18%, and 43%, respectively (Figure 3B). Since Ca^{2+} is more effective than K^+, divalent metal ion is apparently important. Since spermidine does not mimic the effects of Ca^{2+}, divalent metal ion is probably acting via a specific interaction within the transpososome rather than by nonspecific charge neutralization.

Physical Detection of Noncovalent Cocomplexes between Transpososomes and Target DNA

Radiolabeled DEB complexes were incubated with target DNA exactly as during Stage I of a target commitment experiment and then analyzed by gel electrophoresis directly, without addition of Mg^{2+} and without removal of proteins. In such experiments, 80%–100%

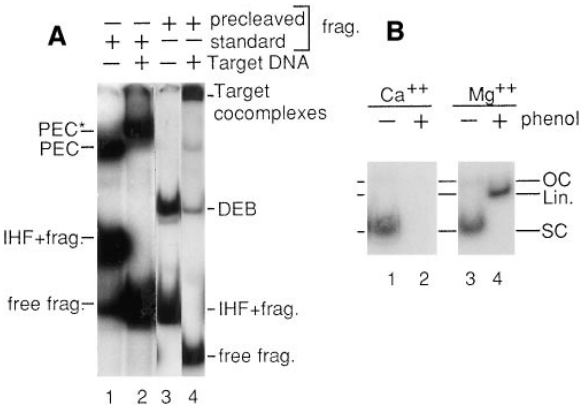

Figure 4. Physical Association of Synaptic Complexes with Target DNA

(A) PEC and DEB complexes were assembled with the standard substrate (lanes 1–2) or the precleaved-end substrate (lanes 3 and 4), respectively, which were both radiolabeled. After assembly was complete, reaction mixtures were supplemented to 4 mM $CaCl_2$, and supercoiled plasmid target DNA was added to samples, as indicated, and further incubated for several hours. Reactions were analyzed by electrophoresis through a native polyacrylamide gel. Association of DEB complexes with the target DNA is indicated by the presence of a characteristic curved band of radiolabel at the position of the closed circular plasmid DNA, close to but clearly below the label trapped in the wells of the gel (seen in lighter exposures of lane 4). Essentially all DEBs are present in this species; few remain at the position of uncomplexed DEBs (compare lanes 4 and 3). PEC complexes exhibit essentially no complexes with target DNA; material at the top of the gel does not exhibit the characteristics of cocomplexes (see above) and thus represents trapping of other species in the well. Also, in the presence of target DNA (lane 2), PEC complexes occur in an alternative conformer, PEC*, which lacks IHF and migrates at a new position just above the PEC band owing to unfolding of the DNA arms within the complex (Sakai, 1996).

(B) DEB complexes were incubated with a target DNA in the presence of 4 mM $CaCl_2$ (lanes 1 and 2) or 4 mM $MgCl_2$ (lanes 3 and 4) and incubated for 3 hr. Reactions were loaded onto a 1% agarose gel either directly (lanes 1 and 3) or after the addition of phenol (lanes 2 and 4). Results identical to lanes 2 and 4 are also obtained by addition of SDS to 4%. OC, Lin, and SC indicate the positions of nicked, linear, and supercoiled target plasmid DNA, respectively. Small amounts of label occur at the position of nicked circles owing to interaction of transpososomes with small contaminating levels of this form present in the supercoiled plasmid preparation prior to any reaction. The level of cocomplexes observed with Ca^{2+} (lane 1) varies from 80%–100% of the level observed with Mg^{2+} (lane 3) among different experiments.

of DEBs comigrate with supercoiled target DNA in both acrylamide and agarose gels (Figure 4A, compare lanes 4 and 3; Figure 4B, lane 1). The observed association is noncovalent. Treatment of such a reaction mixture with phenol (Figure 4B, lane 2) or SDS (data not shown) eliminates the slowly migrating radiolabeled species representing DEB–target DNA cocomplexes and releases free radiolabeled substrate fragment (data not shown). Such samples give efficient strand transfer upon addition of Mg^{2+} (Figure 4B, lane 4).

Nearly all DEB complexes are found in cocomplexes by gel electrophoresis, while only ~50% exhibit target commitment. Thus, in the target commitment protocol, at the end of Stage I, ~40% DEBs are present in noncovalent DEB–target DNA cocomplexes that do not ultimately proceed to strand transfer.

PEC complexes behave very differently from DEB complexes. Essentially no PEC–target DNA cocomplexes are detectable by gel electrophoresis, and incubation with target DNA does not significantly reduce the number of PECs that run (Figure 4A, lanes 1 and 2). Synaptic complexes in which one or both transposon ends are nicked also do not form cocomplexes in this assay (Bolland and Kleckner, 1996).

SEB complexes have not been analyzed for target association as extensively as other types of transpososomes but appear to associate with target DNA as efficiently as DEB complexes, or nearly so (Sakai, 1996).

Thus, SEB cocomplexes occur at high levels, while target commitment is weak or absent (see above). Similarly, when K^+ is the only cation present in the reaction mixture, DEBs still form cocomplexes (Sakai, 1996) but exhibit poor commitment (see above). Finally, DEB transpososomes formed on transposon-end substrates having certain types of single-strand extensions at the transposon termini also exhibit robust physical association without detectable target commitment (Sakai, 1996). We infer target commitment has more stringent requirements than physical association, with divalent metal ion being one differentially required feature.

These findings are consistent with the existence of two types of cocomplexes, a weaker earlier type and a stronger later type. Both types of cocomplexes would be physically detectable (~90%), while only the second type would give target commitment (~50%). Similarly, stringent situations might permit formation of the first type while precluding (conversion to) the second type. Alternatively, however, a single type of cocomplex might, upon addition of Mg^{2+}, have a roughly equal probability of either dissociating or proceeding to strand transfer, and physical associations observed under stringent conditions might not represent a normal interaction.

Gel electrophoresis analysis has revealed that strand-transfer complexes examined without deproteinization comigrate with unreacted target DNA (Figure 4B, lane 3), even though the component DNA product is a linear species containing terminal single-stranded gaps. Thus, strand transfer occurs without bulk loss of supercoils in the target molecule. Also, strand-transfer junctions remain closely held within the synaptic complex in such a way that the protein–protein interactions of the synaptic complex keep target DNA duplex from rotating around the point of discontinuity. In Mu strand-transfer complexes, in contrast, target DNA supercoiling is lost (Surette et al., 1987; Mizuuchi, 1992).

Decay of DEB–Target DNA Cocomplexes

In the Presence of Ca^{2+}

To examine dissociation of DEB–target DNA cocomplexes, radiolabeled DEBs were incubated with Target 1 for 4 hr in the presence of Ca^{2+}. At this point, with t = 0, an equal amount of Target 2 was added to the reaction as a challenge. The distribution of DEB complexes between the two target DNA species as a function of time was then assayed in an agarose gel. The conditions and

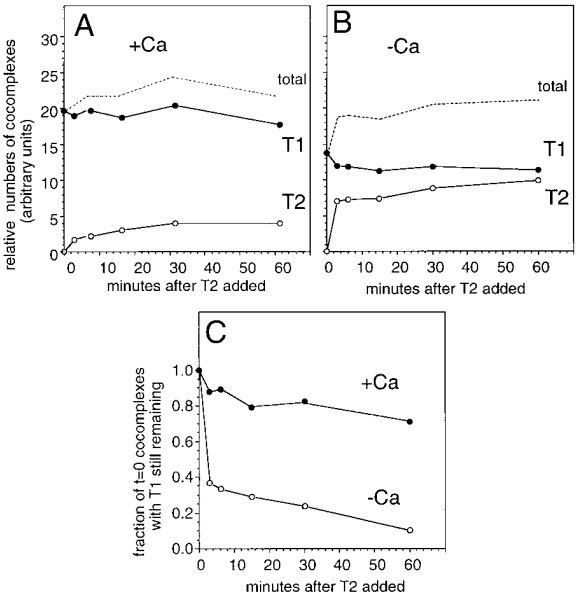

Figure 5. Stability of the DEB–Target Cocomplexes

DEB complexes were assembled in parallel in two separate but identical reaction mixtures under standard conditions. After assembly, the reaction in (A) was supplemented to 4 mM $CaCl_2$, while reaction in (B) was not. Both reactions were then incubated with 1 μg of the 3 kb target plasmid DNA (T1) for 3 hr. At the end of this period, essentially all active DEB complexes are associated with target DNA. Finally, at t = 0, 1 μg of the 5 kb target plasmid was added to each reaction (T2). Aliquots were withdrawn at indicated times and loaded directly onto a 1% agarose gel under tension; t = 0 sample was taken prior to addition of T2.

(A and B) The relative amounts of radioactivity that comigrate with each of the two target DNAs are plotted (in arbitrary units) for the reaction containing Ca^{2+} (A) and the standard reaction (B).

(C) Percentage of DEB–T1 cocomplexes present at t = 0 that remain at each time point for experiments in (A) and (B) (see text).

two stages of this experiment are exactly analogous to those of the target commitment analysis, except that here no Mg^{2+} is added along with Target 2.

The vast majority of DEB complexes are associated with target DNA after addition of Target 1 (as above). However, the total number of cocomplexes (Target 1 + Target 2) does increase by an additional 15% after addition of Target 2, presumably in response to the new higher target DNA concentration (Figure 5A). Also, DEB complexes redistribute somewhat between the two target species; still, an equal distribution is not achieved even after 60 min, at which point the ratio of Target 1 cocomplexes to Target 2 cocomplexes is 4.5:1 (Figure 5A). Thus, many DEB complexes become very stably associated with target DNA during Stage I of this experiment.

Decay of the DEB–Target 1 cocomplexes present prior to Target 2 addition can be quantified as a function of time after Target 2 addition if we assume, as in target commitment analysis, that DEB complexes that associate with a target DNA following addition of Target 2 choose randomly between Target 1 and Target 2. In essence, for every Target 2 interaction detected at a given time (t), a new Target 1 interaction must also have occurred after addition of Target 2. Thus, at that particular time, the number of Target 1 interactions remaining from the first phase of the experiment corresponds to the total Target 1 interactions detected less a number equivalent to the number of Target 2 interactions detected. Of the original cocomplexes present at the time of Target 2 addition, a minority appear to decay rather rapidly, while most are still present 60 min later (Figure 5C). These kinetics provide another hint that there may be two types of cocomplexes.

In the Absence of Ca^{2+}

Omission of Ca^{2+} from reaction mixtures containing DEB complexes and target DNA decreases the stability of DEB–target DNA cocomplexes. In this situation, when Target 1 and Target 2 are added in succession as above, a nearly random mixture of Target 1 and Target 2 cocomplexes is observed by t = 60 min after Target 2 addition (Figure 5B), with 90% of the DEB–Target 1 cocomplexes present at t = 0 inferred to have dissociated by this point (Figure 5C). The kinetics of dissociation again hint at biphasic decay. Ca^{2+} likely reduces the dissociation rates in both phases.

Omission, absence, or chelation of Ca^{2+} also reduces the level of DEB–target DNA cocomplexes (e.g., compare t = 0 in Figures 5A and 5B; other data not shown). A higher dissociation rate(s) for cocomplexes presumably contributes to this effect.

Preincubation of DEB Complexes with Target DNA Accelerates the Rate of Strand Transfer

At least 60 min elapses between excision and strand transfer during Tn10 transposition in vivo and under standard conditions in vitro (Haniford et al., 1991; Haniford and Kleckner, 1994; Chalmers and Kleckner, 1996; Kleckner et al., 1996). It seemed possible that establishment of an effective interaction between DEB complexes and target DNA might account for a significant fraction of this time interval.

We therefore asked whether the rate at which DEB complexes carry out strand transfer is or is not influenced by preincubation with target DNA. DEB complexes were assembled on precleaved substrate fragments. Two identical aliquots of the assembly reaction were then supplemented with Ca^{2+} and preincubated either with or without supercoiled target DNA. Strand transfer was then initiated in both reactions by addition of Mg^{2+}; simultaneously, target DNA was added to the reaction not already containing it. The level of strand-transfer products was then determined as a function of time after Mg^{2+} addition by withdrawing aliquots, stopping the reaction by phenol extraction, and analyzing labeled DNA products. Both DEST and single-end strand-transfer (SEST) products were examined. SESTs occur at detectable levels at intermediate times when Ca^{2+} is present along with Mg^{2+}, as here. By the end of such an experiment, with or without preincubation, essentially all DEB complexes present have undergone strand transfer at one or both ends (data not shown), and nearly all products are DESTs (Figure 6).

Preincubation of DEB complexes with target DNA dramatically accelerates the appearance of strand-transfer products (Figure 6). The kinetics with which DEB complexes undergo their first strand-transfer event are given by the sum of SESTs plus DESTs plotted as a function

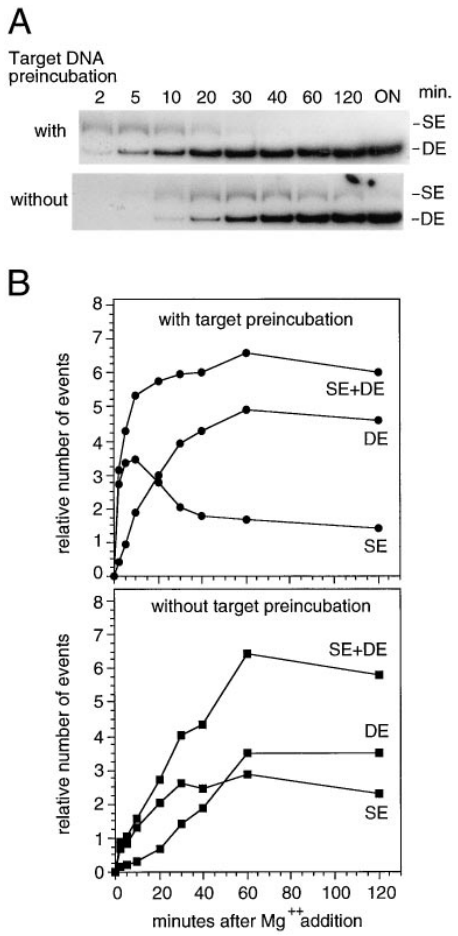

Figure 6. Preincubation of DEB Complexes with Target DNA Accelerates the Rate of Strand Transfer

DEB complexes were assembled under standard conditions, and the reaction mixture was adjusted to 4 mM $CaCl_2$. Two identical aliquots of the mixture were then further incubated in the presence or absence of supercoiled target DNA. At this point (t = 0), both reactions were adjusted by addition of 4 mM $MgCl_2$, and target DNA was added to the reaction from which it was absent. Aliquots were withdrawn at indicated time points; reactions were stopped and proteins were removed by phenol extraction.

(A) Strand-transfer products were analyzed by electrophoresis through a 5% native polyacrylamide gel. DEST (double-end strand transfer), in which both transposon ends are joined to the target DNA, yields a labeled linearized target plasmid. SEST (single-end strand transfer), in which only one transposon end is covalently joined to the target plasmid, yields a labeled nicked circular target plasmid.

(B) Relative number of SEST and DEST products observed in the two reactions. Values are plotted in arbitrary units obtained by densitometric analysis of the data in (A), except that DEST values have been normalized to account for the fact that DEST products contain twice as much label as an SEST product. Also shown is the occurrence of the first strand-transfer event of each transpososome, given by the relative number of cases in which strand transfer has occurred at one or both ends, i.e., the sum of the SEST and DEST events.

of time. With preincubation, 50% of the DEB complexes that carry out strand transfer (i.e., ~50% of all DEB complexes; see above) have done so at at least 1 end within 2 min after addition of Mg^{2+}; 90% have done so within 15 min. In the absence of preincubation, ~25 and ~50 min are required to achieve these same levels. In the preincubated sample, occurrence of the first strand transfer fits a forward rate constant of $k_f = 0.35\ min^{-1}$.

The precise basis for this effect of target DNA preincubation on appearance of strand-transfer products remains to be determined. Additional studies suggest that about half of the observed acceleration probably results from an indirect effect of plasmid DNA addition that precedes any target interaction, plus an apparently short time required thereafter for cocomplex formation per se. Addition of plasmid DNA titrates IHF out of DEB complexes, thereby triggering a conformational change that is required for, and followed closely by, cocomplex formation, which is 50% complete by ~12 min after target DNA addition. (Sakai, 1996; unpublished data). The remaining half of the acceleration should thus reflect time that, in the absence of preincubation, could be required either for a transition from an initial type of cocomplex to a second more stable type of cocomplex and/or for occurrence of a conformational change that is subsequent to all target DNA interactions. Strand transfer would follow rapidly in either case (see above).

This latter effect, whatever its precise basis, may well be of primary importance during normal, i.e., unstaged, transposition reactions. The conformational change that precedes cocomplex formation, while it delays strand transfer in the current protocol, is likely to be irrelevant in unstaged reactions, where it probably normally occurs immediately after PEC formation, prior to or concomitant with appearance of full-excision products (Sakai, 1996). Furthermore, in unstaged in vitro reactions, strand transfer occurs with the same kinetics for both inter- and intramolecular target sites, even though the effective target DNA concentration is at least 100-fold lower in the former case than in the latter (Chalmers and Kleckner, 1996); similar kinetics are also observed in vivo (see above). Thus, also, the rate-determining step in conversion of DEBs to strand-transfer products in a normal reaction is probably not the initial coming-together of DEBs and target DNA, which should depend strongly on target DNA concentration. Therefore, with the two earlier events excluded, the most likely candidate(s) for the slow step(s) in conversion of excised transposons to strand-transfer product during a normal transposition reaction should be an event(s) subsequent to initial cocomplex formation between a DEB and a target DNA, as observed here.

Discussion

Tn10 Synaptic Complexes Can Capture Target DNA only after Transposon Excision

Tn10 DEB synaptic complexes undergo physically detectable and functionally significant interactions with target DNA prior to strand transfer. PEC complexes, in contrast, exhibit no such interactions. Also, synaptic complexes containing nicked ends exhibit no physical interaction. These findings strongly suggest that target DNA normally enters the Tn10 transposition reaction only after double-strand cleavage at the transposon ends. SEB complexes exhibit an intermediate behavior that, given that SEBs are very transient intermediates

(Haniford and Kleckner, 1994), is likely not very significant biologically.

Tn10/IS10 Differs from Mu and Tn7 with Respect to the Timing of Target Interaction

Tn10's ability to select a target DNA only after transposon-end cleavage distinguishes this element from previously analyzed transposons, i.e., Mu and Tn7, which both select a target DNA prior to cleavage (see Introduction). The biological rationale for this difference may be as follows.

Mu and Tn7 constrain their target site selection via a transposition-immunity mechanism that precludes (often suicidal) utilization of target sites very near the ends of the transposon itself (Adzuma and Mizuuchi, 1988; Craig, 1996a, 1996b). Dependence of cleavage upon a prior effective target DNA interaction helps ensure that no reaction occurs until inappropriate target loci have been excluded.

For Tn10/IS10, in contrast, promiscuous target site selection is advantageous. IS sequences often occur in composite transposons such as Tn10, flanking a determinant(s) of interest, and formation of a composite transposon of reasonable size requires insertion of two IS elements very near to one another. Thus, target immunity, which precludes utilization of target DNA sites located within 290 and ≥20 kb for Tn7 and Mu, respectively (DeBoy and Craig, 1996; P. Higgins, personal communication), would be deleterious. Moreover, insertion of one composite element into itself is one important mechanism by which new composite transposons arise (Kleckner et al., 1996). And since a single IS element is very small, the risk of suicidal insertion is minimal in that case. Correspondingly, Tn10/IS10 does not exhibit target immunity (e.g., Chalmers and Kleckner, 1996).

Notably, Mu transposition is replicative while Tn7 transposition, like that of Tn10, is nonreplicative (Craig, 1996b). Thus, differences in the relative timing of target interaction and transposon-end cleavage do not appear to be motivated primarily by the type of transposition mechanism.

A reviewer points out, however, that since Mu identifies its target DNA while still attached to its donor site, it might never utilize a distant target site unless utilization of nearby sites was precluded. If so, development of such a transposon might be codependent upon development of target immunity and thus indirectly promote development of early target interaction.

Conversely, late target interaction could help assure nonreplicative Tn10/IS10 transposition. A Tn10 transpososome containing transferred-strand nicks at both ends of the element is chemically competent to carry out strand transfer but does not do so. Dependence of target interaction upon double-strand cleavage could be one reason (Bolland and Kleckner, 1995). Indeed, during Tn7 transposition, with early target DNA interaction, a mutational defect in catalysis of nontransferred-strand cleavage permits Tn7-promoted cointegrate formation (Gary et al., 1996; May and Craig, 1996). Thus, for wild-type Tn7, cointegrate formation may be precluded solely by the high efficiency of nontransferred-strand cleavage.

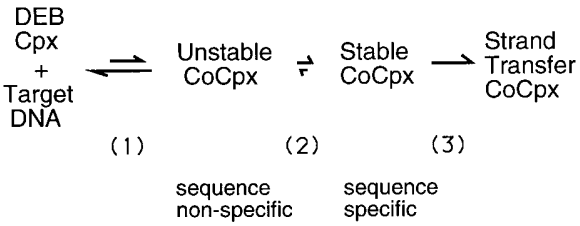

Figure 7. Model for Interaction of DEB Complexes with Target DNA

(Step 1) A DEB transpososome and a target DNA molecule interact to form an unstable cocomplex. The relevant interaction is proposed to be sequence nonspecific with respect to the target DNA molecule, with DEB complexes moving by one-dimensional diffusion around the point of initial contact.

(Step 2) The unstable cocomplex is converted to a stable cocomplex. This step is rate determining for conversion of DEB synaptic complexes to covalent strand-transfer complexes. Within this stable form of cocomplex, the DEB transpososome is proposed to be locked in at a specific target DNA site. Target specificity determinants would determine the array of insertion sites observed in a population of resultant strand-transfer complexes by influencing the forward and/or backward rate constants for Step 2. Divalent metal ion (Ca^{2+} in these experiments but normally Mg^{2+}) appears to exert its effects on DEB–target DNA interactions primarily by increasing the dissociation rate for the cocomplexes formed at this step, with secondary effects on other steps.

(Step 3) Strand transfer. A covalent joint is formed between a 3′ OH terminus and a target DNA strand at one end of the transposon (followed closely by strand transfer at the second end; data not shown).

Mechanistic Considerations

A Two-Stage Model for Noncovalent DEB–Target DNA Interactions

We propose that DEB complexes interact with target DNA via a two-step process in which two qualitatively different types of DEB–target DNA cocomplexes form in succession (Figure 7). The initial interaction between a DEB transpososome and a target DNA molecule would yield a relatively unstable cocomplex, which would then be converted to a second more stable type of cocomplex that in turn would undergo covalent strand transfer. In the simplest case, the rate-determining step for the appearance of strand-transfer products from DEB complexes would be conversion from the first type of cocomplex to the second type.

Evidence consistent with or suggestive of the existence of two types of cocomplexes, and indications that the second type could be rate determining for strand transfer, have been detailed above. Also, preliminary best-fit biphasic decay curves for the data in Figure 5, taken together with known concentrations of the starting components and the apparent rate with which preincubated DEB complexes undergo strand transfer, yield satisfactory estimated kinetic constants for the proposed reaction.

Additional features of the model can be suggested based on the fact that Tn10 is known to insert preferentially at particular hot spots, owing to the combined effects of a 6 bp consensus sequence plus presumptively structural effects of base pairs immediately flanking the consensus (Bender and Kleckner, 1992; Kleckner et al., 1996; also, see Hallet et al., 1994). The first type of DEB–target DNA cocomplex could reflect a sequence-nonspecific interaction of DEB complexes and a target

DNA, with concomitant one-dimensional diffusion in the vicinity of the initial contact. The second more stable interaction could then correspond to the locking in of the DEB complex at some particular target site, with the array of target sites selected at this point determined by the effects of insertion-specificity determinants on the stability of that complex (Figure 7). This type of two-stage model has ample precedent in the way in which DNA-binding proteins, e.g., EcoRI and Lac repressor, identify their (sequence-specific) binding sites (e.g., Jack et al., 1982; Terry et al., 1985).

In the context of this model, the major effect of divalent metal ion on DEB–target DNA interactions could be to favor association and/or disfavor dissociation of the second type of cocomplex (Figure 7). This scenario would explain why divalent metal ion accelerates the rate of appearance of strand-transfer products from DEB complexes, is differentially required for target commitment as compared to cocomplex formation, and decreases the dissociation rate(s) of cocomplexes. A special role for divalent metal at this step in the reaction is also attractive because the locking in of a DEB complex at a specific target site, proposed to occur at this step, could be expected to involve a more intimate contact of target DNA with the catalytic active site of transposase, where divalent metal ion is presumed to be sitting. Effects of divalent metal ion on association and/or dissociation rates for the first type of cocomplex are also likely, however (Figure 5).

Interestingly, Junop and Haniford (1997) have recently analyzed cocomplexes formed between DEB complexes and oligonucleotide target DNAs. Their study reports important effects of both divalent metal ion and DDE catalytic residues, further implicating the catalytic active site of transposase in target DNA interactions; also, cocomplexes formed on a known target DNA hot spot are found to be more stable and to have significantly different properties than those formed on target substrates having other sequences. These and other findings reported in that study are readily accommodated by the model proposed here.

Alternative interpretations of individual data are possible. Nonetheless, the model described in Figure 7 is a unifying interpretation that provides an attractive working hypothesis until otherwise proven.

Naked Target Capture

During λ site-specific recombination, the *attP* intasome captures a naked *attB* partner (Richet et al., 1988). Since gel-purified DEB complexes are proficient for target capture, the Tn10 DEB synaptic complex may similarly capture a naked target DNA. Similarly, DEB–target DNA cocomplexes can be obtained with small DNA oligonucleotide target molecules even in the presence of heparin at a concentration (25 ng/μl) expected to titrate any free transposase away from nonspecific association with target DNA (Junop and Haniford, 1997).

Target Capture via the Ends-Binding Pocket

We are attracted to the specific idea that target DNA comes to occupy the same binding pocket within the synaptic complex that was previously occupied by (now-released) flanking donor DNA. First, target DNA must necessarily be very closely juxtaposed to the 3′ OH groups at the two transposon termini (which formerly were attached to the flanking DNA sequences). Second, this economical model fits with indications that a single catalytic active site unit within the synaptic complex carries out one transferred-strand nick, one nontransferred-strand nick and one strand-transfer event (though not necessarily all at the same end) (Bolland and Kleckner, 1995). Third, this model accommodates the formation of a very tight "seal" between the protein and DNA components within the synaptic complex at the strand-transfer step, as indicated by trapping of target DNA supercoils within strand-transfer complexes (see above).

This idea can also be coupled with the model in Figure 7 to explain additional observations. PEC complexes exhibit no physical association with target DNA, SEB complexes exhibit physical association but not commitment, and DEB complexes exhibit both physical association and commitment. Perhaps a single free flanking DNA site is sufficient for stable physical interaction, while a full target DNA-binding pocket is required for a configuration that gives target commitment and strand transfer (e.g., for Steps 1 and 2, respectively, in the model of Figure 7).

Experimental Procedures

Transposon-End Substrates and Target DNAs

Standard transposon end-substrate was a 136 bp SalI–BglII fragment of pNK1935 containing 87 bp of the transposon end and 49 bp of flanking DNA (Sakai et al., 1995). Substrate fragment precleaved at bp1 was generated by BamHI–PvuII restriction digestion of pNK3287 (the mini-Tn10 transposon from pNK862 [Morisato and Kleckner, 1984] cloned into the PvuII site of pBluescript). Substrate fragments were prepared by separation through a 3% NuSieve agarose gel and eluted using glass beads (Bio101). Fragments were labeled by filling in the 3′ ends by AMV reverse transcriptase and [^{32}P]dATP (Sakai et al., 1995). Target DNAs were supercoiled preparations of pBR322-derived plasmids. Small target DNA was pNK2704, a 3.2 kb plasmid derived from pGC1 by insertion of a HisG1 hot-spot fragment (Bender and Kleckner, 1992). Large target DNA, pNK3837, was constructed by insertion of the XbaI–PstI lacI-containing fragment from pET1a (Studier et al., 1990) into the XbaI–PstI backbone of pGC1.

Preparation of Synaptic Complexes

PEC and DEB complexes were formed in reactions containing either the standard substrate or the precleaved substrate, respectively. SEB complexes were formed in reactions containing a 1:1 mixture of the standard and precleaved substrates in which either the standard or the precleaved fragment was radiolabeled. Complexes were assembled in standard-reaction buffer (22 mM Tris [pH 7.5], 1 mM TES [pH 7.5], 11.5 mM DTT, 19% glycerol, 23 mM NaCl, 100 mM KCl, 0.05 mg/ml BSA, 0.05 mM EDTA); this buffer is referred to throughout this work as "standard conditions." Each reaction contained ~10 fmol of transposon-end fragment (approximately 100,000 cpm), ~40 fmol of transposase, and ~80 fmol of integration host factor (IHF) in a total volume of 20 ml. Reactions were incubated at room temperature (approximately 20°C) for 3 hr, by which time assembly had proceeded to its limit. In such mixtures, 30%–50% of substrate fragment is assembled into defined synaptic complexes; nearly all of the remainder is complexed with IHF (Sakai et al., 1995).

Purification of Synaptic Complexes

Synaptic–complex assembly mixtures were subjected to electrophoresis in a 5% (29:1) polyacrylamide gel. Gel bands containing complexes were identified by autoradiography, excised, mixed with 50 μl standard-reaction buffer and incubated at room temperature for ≥24 hr. Fifteen to forty percent of the radiolabel elutes as intact

complexes (at 1/3 to 1/5 the concentration in primary assembly mixtures); remaining label is present as free DNA.

Target Commitment Assay

For assaying target commitment in assembly mixtures, the standard-assembly reaction was scaled up 3-fold (to 60 μl). Reactions were then supplemented to 4 mM $CaCl_2$ or as indicated (Figure 2). Each such mixture was then split into 3 equal aliquots to which appropriate target DNA(s) was added (1 μg each). After further incubation of mixtures for 4 hr at room temperature, the second target DNA (1 μg) was added if appropriate; at the same time, all reactions were adjusted to 4 mM $MgCl_2$. In these reactions, each type of target DNA molecule is present in a 20- to 30-fold molar excess over transpososome synaptic complexes (5–10 and <.25 nM, respectively). Mixtures were then incubated for an addition period of 12 hr at room temperature, extracted with SDS or phenol–chloroform as indicated, and electrophoresed through a vertical 1% agarose gel. After electrophoresis, gels were dried and exposed to X-ray film. Bands were quantified by Fuji phosphorimager. Purified transpososomes were assayed analogously in reaction mixtures made by addition of appropriate components to tubes containing eluted complexes in reaction buffer (see above).

Calculating the Fraction of Committed Synaptic Complexes

A DEB complex is said to be "committed" if, during Stage II, it undergoes strand transfer to a target DNA selected during Stage I. The percentage of such committed complexes (%TC) can be calculated as follows.

At the end of Stage II, 100% of DEB complexes have undergone strand transfer to one target or the other. For strand transfer to Target 2 (T2), the target DNA was selected during Stage II. For strand transfer to Target 1 ($T1_{total}$), the target DNA might have been selected either during Stage I ($T1_I$) or during Stage II ($T1_{II}$), i.e., $(T1_{total}) = (T1_I) + (T1_{II})$. Furthermore, the ratio of $(T1_{II})/(T2)$ is the same as the ratio of the two target DNAs in the reaction $(T1_{in}/T2_{in})$, because the number of target DNAs is large, as compared to the number of DEB complexes, and target DNAs are chosen at random during Stage II. Thus, $(T1_{II}) = (T2)(T1_{in}/T2_{in})$ and thus, $(T1_I) = (T1_{total}) - (T2)(T1_{in}/T2_{in})$. Moreover, essentially all DEB complexes undergo strand transfer during Stage II. Therefore, among all DEB complexes, the percentage that undergo commitment at the end of Stage I (%TC) = $(T1_I)/(T1_{total}) + (T2)$. Correspondingly, (%TC) = $[(T1_{total}) - (T2)(T1_{in}/T2_{in})]/(T1_{total}) + (T2)$.

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